IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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Applicant: Fenn et al
Serial No: 08/890,490
Filed: July 09, 1997
Title: Frozen Food Product

Group: 1761
Examiner: C Nessler

Commissioner of Patents & Trademarks
Washington DC 20231

Declaration under 37CFR 1.132

I, Christopher Michael Sidebottom, residing at 35, Waterloo Rd, Bedford, Bedfordshire, MK40 3PQ, UK declare and say:

1. I have a Bsc (Hons) degree in Biochemistry from the University of Bristol and a DPhil degree in Chemistry from the University of York. I have been employed as a Research Scientist at Unilever Research, Colworth House, Sharnbrook, Bedfordshire MK44 1LQ, UK since 1981.

2. I am familiar with US patent application having the serial number 08/890,490.

3. US patent application having the serial number 08/890,490 teaches a frozen confectionery product comprising AFP, wherein the ice crystals in the product have an aspect ratio of greater than 1.9. Suitable AFPs for inclusion in such frozen confectionery products provide an ice crystal size upon recrystallisation of less than 20μm when determined using the protocol given in Example V of US patent application 08/890,490.

4. I am familiar with patent application number WO 92/22581 from University of Waterloo. WO 92/22581 discloses the isolation of anti-freeze polypeptides from the extracellular spaces of plant cells.

5. I have isolated various proteins from winter rye and tested their activity as an anti-freeze protein using the protocol as given in example V of US patent application 08/890,490.

The leaves from 30 days cold acclimated rye plants were cut into 3 cm lengths and thoroughly washed in distilled water to remove any cell contents. The leaf pieces were patted dry on a paper towel and totally immersed in an extraction medium of 5 mM EDTA, 10mM ascorbic acid, 2 mM caproic acid, 2 mM benzamidine and 1 mM Phenylmethylsulphonyl fluoride (PMSF). They were then vacuum infiltrated in a Buchner flask for 60 minutes after which time the leaves were removed and patted completely dry. They were then arranged lengthways in a cut off plastic syringe barrel and centrifuged gently at 2000 X g for 30 minutes. The apoplastic extract was collected in an eppendorf tube below the syringe.

The apoplastic extract was concentrated 7 times using Amicon ultrafilter with a PM10 membrane. Initial purification was perfomed by loading 50 microlitres of concentrated apoplastic extract onto a size exclusion Superdex 75 PC 3.2/3.0 column (separation range 3-70 kDa) on a SMART separation system, both from Pharmacia. The buffer was Tris/HCl at pH 9.5. Separation was carried out at a flow rate of 50 microlitres per minute and 50 microlitre fractions were collected up to a volume of 2.5 ml and assayed for ice crystal size on recrystallisation as described in Example V of US patent application 08/890,490.

Active fractions were loaded onto a strong anion exchange MonoQ FPLC column from Pharmacia, equilibrated in 50mM Tris/HCl at pH 9.5, and the proteins were eluted using the same buffer with a linear gradient to 0.5M NaCl over 25 minutes and reduced to 0 M

over 15 minutes. Chromotography was carried out at a flow rate of 1 ml per minute and 1 ml fractions were collected. The fractions which were positive in the test as described in Example V of US patent application 08/890,490 were concentrated on a Centrican PM10 centrifugal concentrator at 7000 rps until the volume was reduced to 50 microlitres and loaded for a second time onto the S75 column. The fraction which satisfied the test of Example V of US patent application 08/890,490 was a single peak at approximately 150 mM salt.

This active fraction was separated on SDS PAGE according to Laemmli (1970) using the Biorad mini system. Samples to be analysed by SDS-PAGE were dissolved in SDS-PAGE sample buffer (Laemmli 1970), heated for 5 minutes at 100°C on a dry heating block (Techne) and centrifuged for 3 minutes at 10,000g at room temperature. Samples (10-50µl) were applied to mini-gels (Biorad, 0.75, 1.0 or 1.5mm thickness, 10, 12, 15% acrylamide or 10-20% gradient acrylamide, pre-poured from Biorad) and electrophoretically separated. Separate polypeptides were fixed and stained in the gel either with Coomassie Brilliant Blue (0.1% (w/v) Coomassie Brilliant Blue in acetic acid/methanol/miliQ water (5:4:31, by vol)) or silver stained using the Biorad silver stain kit according to the manufacturer's instructions. Gels were dried between two sheets of Gelair cellophane in a Biorad gelair dryer according to the manufacturer's instructions. Sigma high and low range molecular weight marker kits were used according to the manufacturer's instructions for determination of apparent M_r on SDS-PAGE. This confirmed that the 32kDa fraction was the active fraction.

6. Only a protein having a molecular weight of 32 kDa provides an ice crystal size number average of less than 20µm when tested according to the Example V of US patent application 08/890,490.

7. I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Tile 18 of the United States Code and that such wilful false statements may jeopardise the validity of this application or any patent issued therein.

Signed: Christopher Michael Sidebottom

Dated: